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(21) International Application Number: PCT/CA93/00239 (22) International Filing Date: 7 June 1993 (07.06.93) (30) Priority data: 892,807 5 June 1992 (05.06.92) US (60) Parent Application or Grant (63) Related by Continuation US 892,807 (CIP) Filed on 5 June 1992 (05.06.92) (71) Applicant (for all designated States except US): DALHOUSIE UNIVERSITY [CA/CA]; Halifax, Nova Scotia B3H 3J5 (CA).		(72) Inventors; and (75) Inventors/Applicants (for US only) : BROWN, Robert [CA/CA]; 18 Swanton Drive, Dartmouth, Nova Scotia B2W 2C4 (CA). MEZEI, Michael [CA/CA]; 14 Starling Street, Halifax, Nova Scotia B3M 1V8 (CA). POHAJDAK, Bill [CA/CA]; 83 Shrewsbury Road, Dartmouth, Nova Scotia B2V 2C4 (CA). KIMMINS, Warwick, Charles [CA/CA]; 5865 Balmoral Road, Halifax, Nova Scotia B3H 1A5 (CA). (74) Agents: GRAVELLE, Micheline, L. et al.; Smart & Biggar, 900 - 55 Metcalfe Street, P.O. Box 2999, Station D, Ottawa, Ontario K1P 5Y6 (CA). (81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: USE OF ZONA PELLUCIDA GLYCOPROTEINS FOR IMMUNOCONTRACEPTION		
(57) Abstract A vaccine for the immunocontraception of mammals is described. The vaccine consists of zona pellucida antigens and an adjuvant encapsulated in a liposome delivery system. The liposome delivery system allows for the slow release of antigen resulting in a prolonged immune response. In particular, after a single injection of the vaccine, levels of anti-zona pellucida antibodies were detected for up to 22 months. Thus, the vaccine according to the present invention is effective after a single dose and is therefore very useful in immunocontraceptive protocols.		

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**USE OF ZONA PELLUCIDA GLYCOPROTEINS FOR IMMUNOCONTRACEPTION
FIELD OF THE INVENTION**

The present invention relates to a vaccine composition for the immunocontraception of mammals.

5 **BACKGROUND OF THE INVENTION**

There is a real need for population control in several species of domestic and wild animals. Methods such as surgical sterilization, or more drastically, culling are generally not acceptable or even allowable in most
10 countries. For example, in Canada the culling or harvesting of seals was prohibited in the early 1980's, resulting in an increased seal population from 10,000 in 1978 to approximately 45,000 at present. Increases in harp seal populations have been much greater. Unfortunately, the
15 increasing population of seals is eating away at the diminishing fish stocks which poses serious problems for the fishing industry. The seals also contain parasites such as seal worms that they pass on to the fish. In 1986 it was estimated that the cost to the fishing industry of removing
20 seal worms from fish by hand was upwards of \$30 million a year. Therefore, it is highly desirable to develop an effective form of contraception in mammals, such as seals, in order to effectively control the population growth of certain mammals.

25 One form of contraception for mammals has involved immunocontraception using glycoproteins isolated from the zona pellucida, a covering which surrounds oocytes. The zona pellucida glycoproteins (hereafter referred to as ZP) provide an attachment site for sperm.
30 Immunocontraception with ZP results in the production of antibodies to ZP which cause (a) an alteration of the nature of the ZP membrane of ova, thereby inhibiting sperm entry, (b) an inhibition of implantation of fertilized ova into the uterus and (c) decreased ovarian follicular
35 differentiation (Henderson, C.J., M.J. Hulme and R.J. Aitken. 1988. Contraceptive potential of antibodies to the zona pellucida. J. Reprod. Fert. 83: 325-343).

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Immunocontraception has been induced with both zona pellucida glycoproteins and epitopes of these glycoproteins which have been sequenced, synthesized and coupled to carrier proteins (Millar, S.E., S.M. Chamow, A.W. Baur, C. Oliver, F. Robey and J. Dean. 1989. Vaccination with a synthetic zona pellucida peptide produces long-term contraception in female mice. *Science* 246: 935-938). The use of ZP glycoproteins for immunocontraception has several advantages over other contraception methods. Firstly, ZP glycoproteins are unique to the female reproductive system and therefore, anti-ZP antibodies likely have little or no effect on other tissues. Secondly, the infertility caused by anti-ZP antibodies is reversible, although, hyper-immunization may cause permanent sterility.

Previous studies have shown that in order to effect immunocontraception, multiple injections of ZP were necessary (Kirkpatrick, J.F., I.K.M. Liu and J.W. Turner. 1990. Remotely-delivered immunocontraception in feral horses. *Wildl. Soc. Bull.* 18: 326-330). Multiple injections are clearly not practical for wild populations as it entails recapturing the same wild animal each time an injection is necessary. Multiple injections are also cumbersome in any situation.

Therefore, it is desirable to develop an immunocontraceptive vaccine which would be effective for long periods following a single injection in an efficient delivery system.

Liposomes have been used to carry drugs to sites of inflammation and infection or in some cases tumours. Liposomes are microscopic spheres composed of either a single or multiple concentric bilayer sheets, and range in size from a nanometer to several micrometers in diameter. These bilayer sheets can be formed from a wide variety of phospholipids in varied formulations. Cholesterol can be included in the bilayer in order to increase the bilayer strength and reduce the leakage of materials encapsulated within the entrapped aqueous interior. A vast array of

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compounds can be associated with liposomes, including small molecules, drugs, proteins, and nucleic acids. Liposome-associated compounds can be encapsulated within the aqueous interior of the liposome (i.e. between the bilayer sheets),
5 integrated into the bilayer, or adsorbed or attached to the bilayer surface. The location depends upon the properties of the associating compounds as well as the procedures used for the formation of the liposome.

A liposome based vaccine system has been
10 described for immunization against human malaria (Fries et al, 1992. Liposomal Malaria Vaccine in humans: A safe and potent adjuvant strategy. Proc. Natl. Acad. Sci. USA. 89: 358-362). In this system, a recombinant malaria protein derived from Plasmodium falciparum was encapsulated
15 into liposomes and injected into male volunteers. However the results indicate that at least three injections of the vaccine were required in order to produce an elevated antibody response.

SUMMARY OF THE INVENTION

20 The present invention relates to an immunocontraceptive vaccine preparation which comprises zona pellucida antigens incorporated into a liposome delivery system. The liposome system effects the slow release of antigen resulting in an extended period of antibody production and thereby
25 by an extended period of contraception. Therefore, the present invention provides a method to achieve immunocontraception of mammals using a single injection of zona pellucida glycoproteins.

Accordingly, the invention provides a vaccine
30 composition for the immunocontraception of a mammal which comprises a zona pellucida derived antigen incorporated into a liposome system.

The invention further provides a vaccine composition capable of inducing the production of antibodies to a
35 zona pellucida antigen, said composition comprising a zona pellucida derived antigen incorporated into a liposome system.

In one embodiment of the present invention, the liposom vaccine composition is freeze-dried and incorporated into a BallistiVet® biobullet. Such an embodiment makes the vaccine easier to deliver to the animal.

- 5 In another aspect, the invention provides a method of preventing fertilization in a mammal which comprises administering an effective amount of the above-described vaccine.

DETAILED DESCRIPTION OF THE INVENTION

10 Isolation and purification of zona pellucida glycoproteins

- Zona pellucida glycoproteins were isolated and purified as described by Yurewicz (in Yurewicz, E.C., A.G. Sacco, and M.G. Subramanian, Structural Characterization of the Mr = 55,000 Antigen (ZP3) of Porcine Oocyte
15 Zona Pellucida, The Journal of Biological Chemistry 262: 564-571 (1987)). Porcine ovaries were homogenized and the oocytes recovered from the homogenate by sieving through nylon screens of decreasing pore size (500, 350, 200, 175, 100 and 40 um). The oocytes were homogenized with a glass-
20 teflon apparatus and the homogenate was passed through a 40 um screen to collect the fractured zonae. The fractured zonae were washed with buffer and recollected on the 40 um screen. Zona pellucida glycoproteins were solubilized by incubating the zonae in a water bath (73°C for 20 minutes).
25 The fraction obtained (solubilized intact zona pellucida glycoproteins, SIZP) was shown to be at least 95% pure by comparison to a reference standard of ZP using an ELISA assay.

- ZP3 was purified from SIZP as described by Yurewicz et al (1987). ZP3 is one of three glycoproteins that make up the mammalian zona pellucida. ZP3 is the major macromolecular component of the oocyte zona pellucida and has been shown to be the receptor for sperm.

- 35 Porcine ZP has been used to effect immunocontraception in a variety of mammals. Porcine ZP pellucida was used in the present studies for several reasons. Firstly, a comparison of the reactivity of ZP from five mammalian

Table 1

Effect of Encapsulating ZP in Liposomes
on Anti-ZP Antibody Production in Rabbits

Time (days)	Ag	Anti-ZP titer*											
		a	b	c	d	e	f						
PRE	SIZP	0	0	0	0	0	0	0	0	0	0	0	0
27	SIZP	2	0	12	2	0	0	0	0.5	22	36	0	0.7
55	SIZP	12	3	96	68	2	1	0	0	50	50	0.3	0.6
69	SIZP	24	24	184	130	-	-	-	-	84	85	-	-
69	ZP3	16	15	97	46	-	-	-	-	74	64	-	-

* A value of 100% indicates the serum being tested has the same titer as a pooled standard serum from rabbits rendered infertile by immunization with SIZP.

a - each rabbit was injected with SIZP (20 µg) in 0.5 ml saline.

b - each rabbit was injected first with SIZP (20 µg in a mixture of saline (0.25 ml) and Freund's complete adjuvant (0.25 ml) followed by two boosters of SIZP (20 µg) in a mixture of saline (0.25 ml) and Freund's incomplete adjuvant (0.25 ml).

c - each rabbit was injected with SIZP (20 µg) with muramyl dipeptide (5 µg) in saline (0.5 ml).

d - each rabbit was injected with SIZP (20 µg) encapsulated in liposomes containing phospholipon 90G (Nattermann Phospholipid Co., Cologne, Germany, 0.1 g), cholesterol (0.01 g) and saline (0.5 ml). Liposomes were prepared according to U.S. patent 4,485,054, which is incorporated herein by reference. Liposomal products containing ZP and adjuvants however can be prepared by many other methods presently known and used for manufacturing liposomes.

e - each rabbit was injected with SIZP (20 µg) encapsulated in liposomes as for d (0.25 ml) in Freund's complete adjuvant (0.25 ml).

f - each rabbit was injected with SIZP (20 µg) encapsulated in liposomes as for d (0.5 ml) containing muramyl dipeptide (5 µg).

PRE= preimmunization.

EXPERIMENT 2

Immunization of Captive Seals

Protocol.

Captive female grey seals (13) were divided into
5 six groups of two animals each, except for group d which
comprised three animals. The groups were immunized with a
single injection i.m. as follows:

(a) ZP (15 µg) in liposomes with Freund's complete
adjuvant (FCA)

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- (b) ZP (90 µg) in liposomes with FCA
- (c) ZP (90 µg) in FCA
- (d) ZP (90 µg) in liposomes with TiterMax
- (e) ZP (90 µg) in TiterMax
- 5 (f) ZP (90 µg) in liposomes with BCG adjuvant in water

All animals received an injection having a volume of 1.0 ml. Bacillus Calmette-Guerin or BCG is one component of FCA. Liposomes were formulated as previously described. TiterMax (0.5 ml) was mixed with saline

10 (0.5 ml) containing SIZP or liposomes (0.5 ml) containing SIZP as described by the manufacturer. TiterMax (Cytrx Corporation, Norcross, Georgia) is a new adjuvant system which claims to induce higher titers of antibodies than FCA with a single injection while being less toxic to animals.

species to rabbit antiserum against porcine ZP indicated that pig ZP was recognized best followed by dog ZP while rat and cat ZP reacted poorly (Maresh, G.A. and B.S. Dunbar. 1987. Antigenic comparison of five species of mammalian zonae pellucida. J. Experimental Zoology 244: 299-307). Since seal and dog are closely related, (Berta, A., C.E. Rae, A.R. Wyss, 1989, "Skeleton of the oldest known pinniped *Enaliarctos mealsi*", Science 244: 60-62), anti-porcine ZP antibodies will bind to seal zonae. Secondly porcine ZP can also be easily obtained from slaughterhouse pigs. Thirdly, pigs ovulate multiple oocytes and thus provide a rich source of zona pellucida.

EXPERIMENT 1

Production of Anti-ZP Antibodies in Rabbits

Protocol.

Rabbits were injected i.m. (two rabbits for each treatment, details of each treatment are given in Table 1) as follows (a) ZP in three injections at monthly intervals, (b) ZP with Freund's complete adjuvant plus two boosters with ZP in Freund's incomplete adjuvant at 4 and 8 weeks and (c) ZP with muramyl dipeptide adjuvant (Sigma chemicals). In a second series, rabbits were immunized as above but ZP, plus adjuvant where applicable, were encapsulated in liposomes and in all cases in the second series only a single injection was administered. Preimmunization serum samples were taken from all rabbits. Titers of antibodies were measured by Elisa assay using both SIZP and ZP3 as antigen.

Results.

The results of this study are illustrated in Table 1. Immunization of rabbits with ZP (no adjuvant) raised a low antibody titer after the third injection. A single injection of ZP in liposomes with no adjuvant raised no detectable antibodies. Use of muramyl dipeptide adjuvant did not stimulate antibody production regardless of being encapsulated in liposomes. Use of Freund's adjuvant stimulated antibody production to high levels after 69 days

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when the antigen was administered by 3 injections 4 weeks
apart or as a single injection in liposomes. However,
titers were higher using the former procedure. Measurement
of antibodies directed specifically against ZP 3 indicated
5 that antibody titers were similar after three injections
without the use of liposomes as they were with a single
injection with liposomes.

Therefore, these results indicate that a vaccine
preparation containing ZP in combination with Freund's
10 adjuvant and encapsulated into a liposome system can induce
significant anti-ZP antibody levels for extended periods of
time after a single injection.

Table 2

Anti-ZP Antibody Production by Captive Seals.

Antigen	Anti-ZP titer (% of standard serum) *																	
	Time (months)																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	16	18	20	22	
(a) ZP (15 µg) FCA/liposomes	4	70	60	155	146	141	136	94	34	20	-	-	-	-	-	-	-	
	3	92	166	216	192	182	224	185	150	117	135	95	87	23	35	20	18	
(b) ZP (90 µg) FCA/liposomes	1	66	206	186	74	169	125	103	64	67	59	68	20	10	3	5	5	
	18	78	230	227	230	247	223	164	127	122	115	74	31	14	10	7	4	
(c) ZP (90 µg) FCA	51	253	201	145	53													
	8	200	171	110	35													
(d) ZP (90 µg) TiterMax/ liposomes	3	16	9	2	1													
	7	22	8	2	3													
	25	28	20	7	2													
(e) ZP (90 µg) TiterMax	10	9	25	30	14	32	-	-	-	-	-							
	3	36	22	59	7	20	-	-	-	-	-							
(f) ZP (90 µg) BCG/liposomes	0.1	0.3	0.1	0.1	0	-	-	-	-	-	-	-						
	0.4	0.2	0.1	0	0.1	-	-	-	-	-	-	-						

ZP in this table indicates SIZP

* A value of 100% indicates the serum being tested has the same titer as a pooled standard serum from rabbits rendered infertile by immunization with SIZP.

- = no samples, seals returned to wild.

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Results

The results of the immunization of the seals are shown in Table 2. The 1st and 2nd line of each group refers to results for two seals receiving the same treatment, that is, each formulation was tested in two seals, except for the ZP/TiterMax/liposomes formulation which was tested in three seals. As can be seen from this table, the anti-ZP titer was greatest for groups (a) and (b) wherein zona pellucida was incorporated into a liposome system. Antibody titers were detected up until 22 months from immunization in the three seals from groups (a) and (b). (The fourth seal was returned to the wild at 11 months.) Without liposomes, group (c), antibody titers dropped off after 5 months. Substituting BCG for Freund's complete adjuvant was not effective in eliciting high titers (group f). The new adjuvant system, TiterMax, was also not effective (groups (d) and (e)).

Therefore, the results of this study demonstrate that incorporation of the ZP and adjuvant into a liposome system results in a prolonged anti-ZP response after a single injection.

EXPERIMENT 3

Immunization of Harbour Seals

Twelve harbour seals, divided into three groups of four, were immunized with ZP in a single injection (Table 3). The seals were immunized immediately after giving birth. Samples were taken in the two or three week period until the seals left the pupping area. The results are shown in Table 3. Immunization (with 30 and 90 µg ZP) of harbour seals that had just given birth resulted in anti-ZP titers of only 9% (range 6 to 12%) of immunocontraceptive levels after 3 weeks. As discussed previously, anti-ZP antibodies can act as an immunocontraceptive by inhibiting sperm penetration of ova or by inhibiting implantation of fertilized ova into the uterus. Since seals breed about 3 weeks after giving birth, it is possible that the above levels of anti-ZP antibodies would

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not affect sperm penetration of the oocytes. Immunization directly after giving birth may, however, have some effect on the implantation of the embryo since seals have a delayed implantation of up to three months.

TABLE 3

Production of anti-ZP antibodies by harbour seals immunized with ZP in a single injection at three doses

ZP (µg)	Animal	Anti-ZP titer (expressed as a % of a standard serum) *	
		Time (weeks)	
		2	3
15	F	-	5.9
	F	1.2	0.4
	F	0.7	2.7
	F	4.8	-
30	F	0.9	-
	F	1.8	6.5
	F	-	12.0
	F	18.0	-
90	F	1.1	6.9
	F	0.4	-
	F	4.7	-
	F	1.6	11.0

F = adult female

- = no sample

* A value of 100% indicates the serum being tested has the same titer as a pooled standard serum from rabbits rendered infertile by immunization with SIZP.

5

EXPERIMENT 4Immunization of Grey Seals

Field studies of grey seals on Sable Island have been conducted and are still in progress. Grey seals go to Sable Island every year to have pups and breed and were therefore used in order to study the immunocontraceptive ability of the vaccine preparation. One group of female grey seals (approximately 100 animals) was immunized during the January 1992 breeding season with liposomes in FCA

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(control) while the other group of 100 animals was immunized with ZP in liposomes in FCA. In January 1993 the seals that returned to Sable Island were tested for anti-ZP titers. The results of this study are shown in Table 4.

- 5 As expected, the control group of seals had antibody titers of zero. The experimental group, on the other hand, had appreciable titers. Providing anti-ZP titers persist for 1 year, it is expected that females in the experiment group will not conceive and therefore should not return to Sable
10 Island in 1994.

- It should also be noted that fewer seals in the experimental group returned to Sable Island during the 1993 season (51 of 85 expected) than the control group (76 of 85 expected). These results demonstrate that the liposome
15 vaccine was effective as an immunocontraceptive even in the 1992 breeding season, immediately following administration of the vaccine. Anti-ZP antibodies inhibit implantation of embryos in the uterus. As seals have delayed implantation of about 3 months, antibody production had sufficient time
20 to build up to concentrations which inhibited implantation (Henderson et al, 1988). This explanation is supported by recapture of three seals (#44, #65 and #66) which had been radio tracked. Their sera had anti-ZP titers of 12, 34 and 56%, respectively, 6 months after being immunized. The
25 seals that return to Sable Island in 1994 will be further analyzed.

TABLE 4

Anti-ZP titers (as a % of a standard serum) in grey seals
one year after injection with liposomes/FCA (controls)
or ZP in liposomes/FCA (experimental)

Controls		Experimental	
Seal #	Titer (%)	Seal #	Titer (%)
1	0	2	12
5	0	5	11
7	0	6	17
8	0	7	38
16	0	8	71
19	0	10	6
27	0	11	4
76	0	15	0
77	0	18	19
86	0	19	37
87	0	20	5
88	0	22	41
94	0	27	21
96	0	29	3
103	0	31	2
112	0	33	67
117	0	34	20
		35	27
		39	8
		41	32
		42	4
		47	16
		48	58
		52	5
		53	42
		55	12
		57	25
		61	10
		62	3
		63	4
		65	51
		67	2
		68	4
		70	33
		71	26
		73	2
		74	91
		76	75
		77	2
		78	26
		79	76
		81	77
		84	29
		88	26
		91	5
		93	16
		94	26
		96	49
		101	10
		104	6

* A value of 100% indicates the serum being tested has the same titer as a pooled standard serum from rabbits rendered infertile by immunization with SIZP.

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EXPERIMENT 5

After the filing of the priority application (USSN 07/892,807), the inventors continued their research to develop and improve the previously mentioned liposomal vaccine formulations incorporating zona pellucida (ZP) antigens. One new embodiment involved the incorporation of a freeze-dried liposome containing ZP and adjuvant into a BallistiVet® biobullet. A biobullet is a gelatin capsule adapted to incorporate antigens for the purpose of vaccinating large animals. The biobullet is shot into the animal with a specially designed gun thus avoiding the need to restrain and inject the animals.

Procedure

1. Preparation of Biobullet

The biobullets were obtained from BallistiVet (Minneapolis, MN) and packed with the appropriate vaccine or control formulation as described below.

2. Immunization of Rabbits

Rabbits (two rabbits for each treatment) were injected i.m. (a + b below) or a biobullet was surgically implanted (c below) as follows: (a) ZP (20 µg) was encapsulated in liposomes prepared according to U.S. patent 4,485,054 and the liposomes were suspended in a mixture of saline (0.25 ml) and FCA (0.25 ml); (b) ZP (20 µg) encapsulated in liposomes prepared as above and suspended in a mixture of saline (0.25 ml) and FIA (0.25 ml) and (c) ZP (20 µg) encapsulated in liposomes prepared as above then freeze-dried and the freeze-dried powder suspended in FCA (0.25 ml). This suspension was packed into a biobullet (BallistiVet) and implanted i.m. by surgery.

Results:

The results of this study are shown in Table 5. Administration of ZP in liposomes with FCA produced high anti-porcine ZP antibody titers within one-two months. Replacing FCA with FIA, only marginally reduced anti-ZP antibody production. Use of freeze-dried liposomes with

FCA in a biobullet resulted in an increased and prolonged production of anti-ZP antibodies.

TABLE 5

Effect of adjuvants on the production of anti-ZP antibodies by rabbits immunized with ZP in liposomes or freeze-dried liposomes in biobullets.

Delivery/adjuvant	Titer (% of standard serum)				
	Time (months)				
	1	2	3	4	5
Liposome/FCA	120	131	103	24	16
	38	38	24	17	19
Liposome/FIA	12	26	38	6	-
	91	97	73	18	19
Biobullet/FCA	159	149	215	27	66
	50	196	344	128	98

FCA = Freund's complete adjuvant.

FIA = Freund's incomplete adjuvant.

EXPERIMENT 6

In this experiment, the three seals from Experiment 2 in groups (a) and (b) that were tested up until month 22 for anti-ZP antibodies were given a booster of SIZP (90 µg) in freeze-dried liposomes with Freund's Incomplete Adjuvant (FIA) delivered with a Ballistivet biobullet into the hindquarter of each seal. Serum samples were taken after 4 weeks (month 23), 6 weeks (month 23.5), 8 weeks (month 24) and 12 weeks (month 25) after administration of the booster and anti-porcine ZP titers measured.

Results:

The results are shown in Table 6. A booster administered using a biobullet increased the anti-ZP antibodies to high levels which peaked 6-8 weeks later. It is expected that at least one more serum sample will be taken from these animals before they are released.

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TABLE 6

Antigen	Anti-ZP Titer (% of a standard serum)			
	Time (months)			
	23	23.5	24	25
ZP (15 µg)	-	-		
FCA/lipos	116	159	119	79
ZP (90 µg)	54	59	68	33
FCA/lipos	15	21	41	13

EXPERIMENT 7

The zone pellucida (ZP) antigen used in the previously mentioned seal studies was porcine derived. Therefore in order to demonstrate that antibodies raised in seals to porcine ZP do cross-react with seal oocytes, the following experiment was conducted.

Procedure:

Antibodies from unimmunized harp seals and harp seals immunized with porcine ZP were purified by affinity chromatography using a Protein A column (Pierce Chemical Co., U.S.A.). The two antibody preparations were labelled (^{14}C) by reductive methylation (Jentoft, N. and D.G. Dearborn, 1979, Labelling of proteins by reductive methylation using sodium cyanoborohydride, J. Biol. Chem. 254: 4359-4365). Labelled antibodies were incubated with either seal or porcine oocytes overnight at 20°C . Unbound antibody was removed by centrifugation with washing (2X's) with saline. Labelled antibody bound to oocytes was determined by liquid scintillation counting (LKB racbeta instrument). Oocytes were counted using a microscope and a haemocytometer-like counting chamber.

Results:

The results are shown in Table 7. Seal and porcine oocytes incubated with labelled antibodies from control seals bound only small quantities of antibody (25-52 DPM).

Seal oocytes (93) bound ^{14}C -labelled anti-porcine ZP antibodies, 119 DPM (35.8 ng protein/oocyte) whereas

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- porcine oocytes (96) bound more antibody (250 DPM; 72.9 ng protein/oocyte). This result suggests that about one-half the antibodies produced against porcine ZP in seals recognized and bound to seal ZP. Increasing the number of seal oocytes from 93 to 178 resulted in an increase in the quantity of antibody bound to oocytes (from 119 DPM to 169 DPM) demonstrating all sites on the seal oocytes capable of binding antibody were occupied. Increasing the number of porcine oocytes from 96 to 192, 432 and 864 oocytes resulted in no significant increase in the quantity of ^{14}C -labelled antibody bound (250, 228, 236 and 277 DPM). This result demonstrated that 96 porcine oocytes bound all anti-porcine-ZP antibody present in the serum from the immunized seal.
- These results verify that anti-porcine ZP antibodies bind to seal oocytes and provide evidence that immunocontraception may be explained by the binding of antibodies to the oocyte surface.

TABLE 7

Binding of ^{14}C -labelled anti-ZP antibodies
to pig and seal oocytes

oocytes		Ab from control seal		Ab from immunized seal	
source	number	Ab bound (DPM)	Ab protein bound (ng/oocyte)	Ab bound (DPM)	Ab protein bound (ng/oocyte)
seal	178	38	5.0	189	26.6
seal	93	25	6.3	119	35.8
porcine	864	52	1.4	277	9.0
porcine	432	47	2.6	236	15.3
porcine	192	45	5.5	228	33.3
porcine	96	55	13.6	250	72.9

The Ab from the control seal added to the oocytes contained 2124 DPM (specific activity, 42,309 DPM/mg protein) whereas the Ab from the immunized seal added to the oocytes contained 1672 DPM (specific activity, 35,685 DPM/mg protein).

SUMMARY

The above described experiments demonstrate that incorporating a zona pellucida antigen with a suitable adjuvant into a liposome delivery system results in an

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effective vaccine system for immunocontraception. The studies have shown that a single injection of the vaccine induces levels of anti-zona pellucida antibodies up to 22 months. A single injection vaccine is clearly
5 advantageous for any immunocontraceptive protocol.

Initial studies have shown that incorporating a freeze-dried liposome containing the ZP antigen and adjuvant into a biobullet also induces high levels of anti-ZP antibodies in the seals. Such a delivery system is
10 advantageous since it does not require that the animals be restrained to be injected with the vaccine.

While the above described experiments relate to certain embodiments of the present invention, it is to be appreciated that various modifications can be made to the
15 vaccine composition without departing from the scope and spirit of the invention. For example, while the above described examples relate to porcine ZP3, any suitable and effective zona pellucida antigen, of porcine or other origin, may be employed. In fact, studies have shown that
20 the genes that code for zona pellucida are conserved among mammals. The zona pellucida antigen may also be purified from oocytes or alternatively, a recombinant ZP antigen may be used. Modifications can also be made to the liposome delivery system. In particular, any liposomal system (such
25 as freeze dried, liquid or semi-solid forms) which allows for the slow, controlled release of the antigen is considered within the scope of the present invention. Finally, the adjuvant used can also be modified, as long as it is effective in enhancing the immune response to the vaccine
30 and is suitable for the intended application.

CLAIMS:

1. A vaccine composition for the immunocontraception of a mammal which comprises a zona pellucida derived antigen incorporated into a liposome system.
- 5 2. A vaccine composition according to claim 1 further comprising a suitable adjuvant.
3. A vaccine composition according to claim 1 or 2 wherein said zona pellucida antigen is ZP3.
4. A vaccine composition according to claim 1 or 2
10 wherein said zona pellucida antigen is solubilized intact zona pellucida.
5. A vaccine composition according to claim 1 or 2 wherein said zona pellucida antigen is porcine derived.
6. A vaccine composition according to claim 2 where-
15 in said adjuvant is Freund's adjuvant.
7. A vaccine composition according to claim 1 or 2 suitable for parenteral administration.
8. A vaccine composition capable of inducing the production of antibodies to a zona pellucida antigen, said
20 composition comprising a zona pellucida derived antigen incorporated into a liposome system.
9. A vaccine composition according to claim 8 further comprising a suitable adjuvant.
10. A vaccine composition according to claim 8 or 9
25 wherein said zona pellucida antigen is ZP3.

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11. A vaccine composition according to claim 8 or 9 wherein said zona pellucida antigen is solubilized intact zona pellucida.
12. A vaccine composition according to claim 8 or 9
5 wherein said zona pellucida antigen is porcine derived.
13. A vaccine composition according to claim 9 wherein said adjuvant is Freund's adjuvant.
14. A vaccine composition according to claim 8 or 9 suitable for parenteral administration.
- 10 15. A vaccine composition according to claim 1, 2, 6, 8, 9 or 13 wherein said liposome system is freeze-dried.
16. A vaccine composition according to claim 15 wherein said freeze-dried liposome system is incorporated into a biobullet.
- 15 17. A method of preventing fertilization in a mammal which comprises administering an effective amount of a vaccine composition according to claim 1, 2 or 6.
18. A method according to claim 17 wherein said composition is administered once as a single dose.
- 20 19. A method according to claim 17 wherein said vaccine is administered parenterally.
20. A method according to claim 17 wherein said mammal is selected from seals and rabbits.
21. A method of preventing fertilization in a mammal
25 which comprises administering an effective amount of a vaccine composition according to claim 1, 2 or 6.

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22. A method according to claim 21 wherein the zona pellucida antigen is present in an amount sufficient to produce antibodies to the zona pellucida antigen in the mammal.

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INTERNATIONAL SEARCH REPORT

PCT/CA 93/00239

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 A61K39/00; A61K9/127		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,8 903 399 (ZONAGEN, INC.) 20 April 1989	1,2,5-9, 12-22
Y	see page 5, line 5 - line 17; claims 1,24-27,33,34 see page 18, line 1 - line 5 see page 18, line 12 - line 15 see page 18, line 22 - page 19, line 4 see page 20, line 12 - line 17	3,4,10, 11
Y	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 262, no. 2, 15 January 1987, BALTIMORE US pages 564 - 571 E.C. YUREWICZ ET AL. 'STRUCTURAL CHARACTERIZATION OF THE Mr = 55,000 ANTIGEN (ZP3) OF PORCINE OOCYTE ZONA PELLUCIDA.' cited in the application see the whole document	3,10
<div style="display: flex; justify-content: space-between;"> <div> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"d" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 06 SEPTEMBER 1993		Date of Mailing of this International Search Report 23. 09. 93
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer RYCKEBOSCH A.O.

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	GB,A,1 504 259 (MERCK & CO INC.) 15 March 1978 see the whole document	4,11
A	J.L. BITTLE ET AL. (ED'S) 'VACCINE BIOTECHNOLOGY' 1989 , ACADEMIC PRESS , NEW YORK & pages 301 - 343 A. ALTMAN ET AL. 'IMMUNOMODIFIERS IN VACCINES' see page 323 - page 324	1-22

INTERNATIONAL SEARCH REPORT

International application No.:

PCT/CA93/00239

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 17-22 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

CA 9300239
SA 74932

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

06/09/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8903399	20-04-89	US-A- 4996297	26-02-91
		AU-B- 630862	12-11-92
		AU-A- 2536088	02-05-89
		EP-A- 0396552	14-11-90
		JP-T- 3502571	13-06-91
<hr/>			
GB-A-1504259	15-03-78	None	
<hr/>			

EPO FORM P007

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82